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## (54) Title: NOVEL OSTEOINDUCTIVE COMPOSITIONS

## (57) Abstract

Human and bovine bone inductive factor products and processes. The factors may be produced by recombinant techniques and are useful in the research and treatment of bone and periodontal defects.

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INTERNATIONAL CLASSIFICATION

1. INTERNATIONAL CLAS.

## NOVEL OSTEOINDUCTIVE COMPOSITIONS

The present invention relates to novel proteins and processes for obtaining them. These proteins are capable of inducing cartilage and bone formation.

Background

Bone is a highly specialized tissue characterized by an extensive matrix structure formed of fibrous bundles of the protein collagen, and proteoglycans, noncollagenous proteins, lipids and acidic proteins. The processes of bone formation and renewal/repair of bone tissue, which occur continuously throughout life, are performed by specialized cells. Normal embryonic long bone development is preceded by formation of a cartilage model. Bone growth is presumably mediated by "osteoblasts" (bone-forming cells), while remodeling of bone is apparently accomplished by the joint activities of bone-resorbing cells, called "osteoclasts" and osteoblasts. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See, e.g. European patent applications 148,155 and 169,016 for discussions thereof.

(57) ABSTRACT

Brief Description of the Invention

The present invention provides novel proteins in purified form. Specifically, four of the novel proteins are designated BMP-1, BMP-2 Class I (or BMP-2), BMP-3, and BMP-2 Class II (or BMP-4) wherein BMP is bone morphogenic protein. These proteins are characterized by peptide sequences the same as or substantially homologous to amino acid sequences illustrated in Tables II through VIII below. They are capable of inducing bone formation at a predetermined site. These bone inductive factors are further characterized by biochemical and biological characteristics including activity at a concentration of 10 to 1000ng/gram of bone in an in vivo rat bone formation assay described below. Proteins of this invention may be encoded by the DNA sequences depicted in the Tables or by sequences capable

substantially the same DNA sequence, as depicted in nucleotide #356 through nucleotide #1543 of Table VII. The human peptide sequence identified in Table VII is 396 amino acids in length. hBMP-2 or related bone inductive proteins may also be characterized by at least a portion of this peptide sequence. hBMP-2 Class I is further characterized by the ability to induce bone formation.

The homologous bovine bone inductive protein of the invention designated bBMP-2 Class I (or bBMP-2), has a DNA sequence identified in Table III below which represents the genomic sequence. This bovine DNA sequence has a prospective 129 amino acid coding sequence followed by approximately 205 nucleotides (a presumptive 3' non-coding sequence). bBMP-2, Class I is further characterized by the ability to induce bone formation. A further bone inductive protein composition of the invention is designated BMP-2 Class II or BMP-4. The human protein hBMP-2 Class II (or hBMP-4) is characterized by a variety of osteogenic activities and is further characterized by at least a portion of the same or substantially the same peptide factors have been described. see, e.g., European patent sequence between amino acid #1 through amino acid #408 of Table VIII, which represents the cDNA of hBMP-2 Class II. This peptide sequence is encoded by at least a portion of the same or substantially the same DNA sequence as depicted in nucleotide #403 through nucleotide #1626 of Table VIII. This factor is further characterized by the ability to induce bone formation.

Still another bone inductive factor of the invention, BMP-4, wherein the bovine homolog is bBMP-3. bBMP-3 is characterized by peptide sequences the same as substantially the same as amino acid #1 through amino acid #175 of Table IV A and B. bBMP-3 is further characterized by the ability to induce bone formation. The bovine factor may be employed as a tool for obtaining the analogous human BMP-3 protein or other mammalian bone inductive proteins. The proper characterization of this bovine bone

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sequences of Tables II through VIII, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention. amino acids in 200

Still a further aspect of the invention is a vector containing a DNA sequence as described above in operative association with an expression control sequence. Such vector may be employed in a novel process for producing a bone growth factor polypeptide in which a cell line transformed with a DNA sequence encoding expression of a bone growth factor polypeptide in operative association with an expression control sequence therefor, is cultured. This claimed process may employ a number of known cells as host cells for expression of the polypeptide. Presently preferred cell lines are mammalian cell lines and bacterial cells. and 10 more. Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

Detailed Description of the Invention and 10 the same page. The proteins of the present invention are characterized by amino acid sequences or portions thereof, the same as or substantially homologous to the sequences shown in Tables II - VIII below. These proteins are also characterized by the ability to induce bone formation. and 10 further. The bone growth factors provided herein also include factors encoded by the sequences similar to those of Tables III - VIII, but into which modifications are naturally provided (e.g. allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Tables II - VIII. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with bone growth factor polypeptides of Tables II - VIII may possess bone growth factor biological properties in common therewith. Thus, they may be

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or disulfide linkages, with the sequences of Tables II - VIII and encodes a bone growth factor having one or more bone growth factor biological properties clearly encodes a member of this novel family of growth factors, even if such a DNA sequence would not stringently hybridize to the sequence of Tables II or VIII.

Similarly, DNA sequences which code for bone growth factor polypeptides coded for by the sequences of Tables II - VIII, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel growth factors described herein. Variations in the DNA sequences of Tables II - VIII which are caused by point mutations or by induced modifications to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention. and preferred embodiments thereof.

Another aspect of the present invention provides a novel method for producing the novel osteoinductive factors. The method of the present invention involves culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding for expression for a novel bone growth factor polypeptide of the invention, under the control of known regulatory sequences. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, *Nature*, 293:620-625 (1981), or alternatively, Kaufman et al, *Mol. Cell. Biol.*, 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. A similarly useful mammalian cell line is the CV-1 cell line.

Bacterial cells are suitable hosts. For example, the

fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. An osteogenic factor of the invention may be valuable in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. Of course, the proteins of the invention may have other therapeutic uses.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to bone defects or periodontal diseases. Such a composition comprises a therapeutically effective amount of at least one of the bone inductive factor proteins of the invention. The bone inductive factors according to the present invention may be present in a therapeutic composition in admixture with a pharmaceutically acceptable vehicle or matrix. Further therapeutic methods and compositions of the invention comprise a therapeutic amount of a bone inductive factor of the invention with a therapeutic amount of at least one of the other bone inductive factors of the invention. Additionally, the proteins according to the present invention or a combination of the proteins of the present invention may be co-administered with one or more different osteoinductive factors with which it may interact. Further, the bone inductive proteins may be combined with other agents beneficial to the treatment of the bone defect in question. Such agents include, but are not limited to various growth factors. The preparation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

In particular, BMP-1 may be used individually in a

applications.

The choice of material is based on, for example, biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. Similarly, the application of the osteoinductive factors will define the appropriate formulation. Potential matrices for the osteoinductive factors may be biodegradable and chemically defined, such as, but not limited to calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyanhydrides; biodegradable and biologically well defined, such as bone or dermal collagen, other pure proteins or extracellular matrix components; nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics; or combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics might also be altered in composition, such as in calcium-aluminate-phosphate and processing to alter for example, pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of such a growth factor, e.g., amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the composition of BMP's. The addition of other known growth factors, such as IGF 1 (insulin like growth factor 1), to the final composition, may also effect the dosage. Generally, the dosage regimen should be in the range of approximately 10<sub>3</sub> to 10<sup>6</sup> nanograms of protein per gram of bone weight desired. Progress can be monitored by periodic assessment of bone growth and/or repair, e.g. x-rays. Such therapeutic compositions are also presently valuable for veterinary applications due to the lack of species specificity.

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are collected.

The unbound fractions are concentrated and dialyzed against 50mM NaAc, 50mM NaCl (pH 4.6) and 6M urea. The unbound fractions are applied to a carboxymethyl cellulose column. Protein not bound to the column is removed by extensive washing with starting buffer, and the bone inductive factor containing material desorbed from the column by 50mM NaAc, 0.25mM NaCl, 6M urea (pH 4.6). The protein from this step elution is concentrated 20- to 40- fold, then diluted 5 times with 80mM KPO<sub>4</sub>, 6M urea (pH 6.0). The pH of the solution is adjusted to 6.0 with 500mM K<sub>2</sub>HPO<sub>4</sub>. The sample is applied to an hydroxylapatite column (LKB), equilibrated in 80mM KPO<sub>4</sub>, 6M urea (pH 6.0) and all unbound protein is removed by washing the column with the same buffer. Bone inductive factor activity is eluted with 100mM KPO<sub>4</sub> (pH 7.4) and 6M urea. Finally, the protein is concentrated approximately 10 times, and solid NaCl added to a final concentration of 0.15M. This material is applied to a heparin - Sepharose column equilibrated in 50mM KPO<sub>4</sub>, 150mM NaCl, 6M urea (pH 7.4). After extensive washing of the column with starting buffer, a protein with bone inductive factor activity is eluted by 50mM KPO<sub>4</sub>, 700mM NaCl, 6M urea (pH 7.4). This fraction is concentrated to a minimum volume, and 0.4ml aliquots are applied to Superose 6 and Superose 12 columns connected in series, equilibrated with 4M GuCl, 20mM Tris (pH 7.2) and the columns developed at a flow rate of 0.25ml/min. The protein demonstrating bone inductive factor activity has a relative migration corresponding to approximately 30,000 dalton protein. The above fractions are pooled, dialyzed against 50mM NaAc, 6M urea (pH 4.6), and applied to a Pharmacia MonoS HR column. The column is developed with a gradient to 1.0M NaCl, 50mM NaAc, 6M urea (pH 4.6). Active fractions are pooled and brought to pH 3.0 with 10% trifluoroacetic acid (TFA). The material is applied to a 0.46 x 25cm Vydac C4 column in 0.1% TFA and the column developed with a gradient to 90% acetonitrile, 0.1% TFA.

the collector.

B. Isoelectric Focusing: are concentrated and dialyzed against 50mM Tris isoelectric point of bone-inductive factor activity is determined in a denaturing isoelectric focusing system. The Triton X100 urea gel system (Hoeffer Scientific) is modified as follows: 1) 40% of the ampholytes used are Servalyte 3/10; 60% are Servalyte 7-9. 2) The catholyte used is 40mM NaOH. Approximately 20ug of protein from Example I is lyophilized, dissolved in sample buffer and applied to the isoelectrofocusing gel. The gel is run at 20 watts, 10°C for approximately 3 hours. At completion the lane containing bone-inductive factor is sliced into 0.5 cm slices. Each piece is mashed in 1.0ml 6M urea, 5mM Tris (pH 7.8) and the samples agitated at room temperature. The samples are acidified, filtered, desalting and assayed as described above. The major portion of activity as determined in the assay described in Example III migrates in a manner consistent with a pI of 8.8 and 9.2<sup>d</sup> to a heparin - Sepharose column prepared in 50mM KPO<sub>4</sub>, 150mM NaCl, 6M urea (pH 7.4). After elution each subunit characterization buffer, a protein with bone-inductive subunit composition of bone-inductive factor is also determined.<sup>7</sup> Pure bone-inductive factor is isolated from a preparative 15% SDS gel as described above. A portion of the sample is then reduced with 5mM DTT in sample buffer and re-electrophoresed on a 15% SDS gel. The approximately 30kd protein yields two major bands at approximately 20kd and 18kd, as well as a minor band at 30kd. The broadness of the two bands indicates heterogeneity caused most probably by glycosylation, other post-translational modification, proteolytic degradation or carbamylatation. The column is developed with a gradient of 0.1M NaAc, 6M urea (pH 7.4). EXAMPLE IIIIC are provided below.

#### Biological Activity of Bone Inductive Factor

A rat bone formation assay according to the general procedure of Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A.,

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above, followed by autoradiography. Analysis reveals a correlation of activity with protein bands at 28 - 30kd and a pI 9.0. An extinction coefficient of 1 OD/mg-cm<sup>2</sup> is used as an estimate for protein and approximating the purity of bone inductive factor in a particular fraction. In the in vivo rat bone formation assays for dilutions as described above, the protein is active in vivo at 10 to 200ng protein/gram bone to probably greater than 1ug protein/gram bone.

EXAMPLE IV

## Bovine Bone Inductive Factor Protein Composition

The protein composition of Example IIA of molecular weight 28,000-30,000 daltons is reduced as described in Example IIIC and digested with trypsin. Eight tryptic fragments are isolated by standard procedures having the following amino acid sequences: filtered, desalting and assayed as described above.

Fragment 1: PARAL G DAI A L D EAE B L G M A N I S T R A T E S  
Fragment 2: A F N Q V E Q Q A A A D L E S A T I A M A N N E R S C H I E L D S

**Fragment 3:** N Y Q D M V V E G

Fragment 4: S T P A Q D V S R

# Fragment of a Note on a Lecture

**Fragment 6:** S D W L S E P H D D P S H T D L E I . Inductive factor is also  
**Fragment 7:** F D R A V C Y N H . Inductive factor is indicated by a dot.  
**Fragment 8:** L P K P E S N ? A T C I Q M S I V E . . . . . . . . . . . . . . . . .  
sample 1A less highly purified preparation of protein from bovine t bone or is prepared according to a purification scheme similar to that described in Example 1A. The purification basically varies from that previously described by omission of the DE-52 column, the CM cellulose column and the mono S column, as well as a reversal in the order of the hydroxylapatite and heparin sepharose columns. Briefly, the concentrated crude 4 M extract is brought to 85% final concentration of ethanol at 4 degrees. The mixture is then centrifuged, and the precipitate redissolved in 50 mM Tris, 0.15 M NaCl, 6.0 M urea. This material is then fractionated on Heparin Sepharose as described. The Heparin bound material

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(a) A [A/G] [A/G] TC [T/C] TC [T/C] TC [A/G] TC [T/C] AA  
(b) A [A/G] [A/G] TC [T/C] TC [T/C] TC [A/G] TC NAG

Bracketed nucleotides are alternatives. "N" means either A, T, C or G.

In both cases the regions of the amino acid sequence used for probe design are chosen by avoiding highly degenerate codons where possible. The oligonucleotides are synthesized on an automated DNA synthesizer; the probes are then radioactively labeled with polynucleotide kinase and  $^{32}\text{P}$ -ATP.

These two sets of probes are used to screen a bovine genomic recombinant library. The library is constructed as follows: Bovine liver DNA is partially digested with the restriction endonuclease enzyme Sau 3A and sedimented through a sucrose gradient. Size fractionated cDNA in the range of 15-30kb is then ligated to the bacteriophage Bam HI vector EMBL3 [Frischauf et al., J. Mol. Biol., 170:827-842 (1983)]. The library is plated at 8000 recombinants per plate. Duplicate nitrocellulose replicas of the plaques are made and amplified according to a modification of the procedure of Woo et al, Proc. Natl. Acad. Sci. USA, 75:3688-91 (1978).

The 32 mer probe is kinased with  $^{32}\text{P}$ -gamma-ATP and hybridized to one set of filters in 5X SSC, 0.1% SDS, 5X Denhardts, 100ug/ml salmon sperm DNA at 45 degrees C and washed with 5X SSC, 0.1% SDS at 45 degrees C. The 17 mer probes are kinased and hybridized to the other set of filters in 3M tetramethylammonium chloride (TMAC), 0.1M sodium phosphate pH 6.5, 1mM EDTA, 5X Denhardts, 0.6% SDS, 100ug/ml salmon sperm DNA at 48 degrees C, and washed in 3M TMAC, 50mM Tris pH 8.0 at 50 degrees C. These conditions minimize the detection of mismatches to the 17 mer probe pool [see, Wood et al, Proc. Natl. Acad. Sci. U.S.A., 82:1585-1588 (1985)]. 400,000 recombinants are screened by this procedure and one duplicate positive is plaque purified. DNA is isolated from a plate lysate of this recombinant bacteriophage designated lambda bP-50. bP-50 was deposited December 16, 1986 with the American

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where  $P_j$  is 20?

TABLE II  
Sequence 280      290      291      292      293      294      295  
CCTTGCTCT TCTCTCTCCA GCT GCC TTC CTT GGG GAC ATC GCC CTG GAC GAG GAG  
Ala Phe Leu Gly Asp Ile Ala Leu Asp Glu Glu

A F L G D I A L D E E

338                    353                    368

GAC TTG AGG GCG TTC GAA GTC CAG QAG GCT CGG GAC CTC AGA CAG CGT GCA ACC  
Asp Leu Arg Ala Phe Gln Val Gln Gln Ala Ala Asp Leu Arg Gln Arg Ala Thr

0 L R A P Q V Q Q A H D L R Q K R A T

383                    398                    (37)                    414                    424

CGC AGG TCT TCC ATC AAA GCT GCA GGTACACTGG GTACAGGCCA  
Arg Arg Ser Ser Ile Lys Ala Ala

R R S S I K A A

is known. Bovine liver DNA is purchased (Sigma) and the precipitation characteristics of the DNA can then be determined by the sucrose gradient. The fractionation may not be linear, so it will be then subjected to the sedimentation analysis (Kleter et al., Pruschaup et al., *J. Virol.*, **1975**, **20**, 101). The virion is plated at 5000 recombinant cells/ml. After 1 hr, nitrocellulose replicas of the plaques are made and assayed according to a modification of the procedure of H. J. Luria, *Proc. Natl. Acad. Sci. USA*, **75**:3688-91 (1978).

The 3k mer probe is kinased with  $\gamma$ -<sup>32</sup>P-ATP and hybridized to one set of filters at 50°C for 16 hrs in Denhardt's liquid, sonicated strips of polyacrylamide gel with 5% SDS, 10% PEG at 4°C overnight, washed, alkali-kinned and hybridized to the other set of filters at 50°C for 16 hrs in Denhardt's liquid, sonicated strips of polyacrylamide gel with 5% SDS, 10% PEG at 4°C overnight. The filters are washed at 50°C for 1 hr in 0.25M NaCl, 0.025M NaHCO<sub>3</sub>, 0.025M Na<sub>2</sub>HPO<sub>4</sub>, 0.025M Na<sub>3</sub>PO<sub>4</sub>, 0.025M Na<sub>2</sub>EDTA, pH 7.0, then exposed to X-ray film at -70°C for 16 hrs.

residue (K) as expected considering the specificity of trypsin. The arginine residue encoded by the CGT triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAG) adjacent to it.

338                          351  
GAC TTG AGG GCC TTC CAA ATG CGC CGT GGT GCG GTC CGA TCA CGT CGG CGT

Goat bBMP-3 is expressed constitutively and predominantly in skeletal muscle. The probes consisting of pools of oligonucleotides are designed on the basis of the amino acid sequences of the tryptic Fragments 9 (Probe #3), 10 (Probe #2), and 11 (Probe #1), and synthesized on an automated DNA synthesizer.

Probe #1: A C N G T C A T [A/G] T T N G G [A/G] T A

Probe #2: C A [A/G] T A [A/G] T A N G C [A/G] T C [A/G] A A

Probe #3: T G [A/G/T] A T N G T N G C [A/G] T G [A/G] T T

A recombinant bovine genomic library constructed in EMBL3 is screened by the TMAC hybridization procedure detailed above in part A. 400,000 recombinants are screened in duplicate with Probe #1 which has been labeled with  $^{32}P$ . All recombinants which hybridized to this probe are replated for secondaries. Triplicate nitrocellulose replicas are made of the secondary plates, and amplified as described. The three sets of filters are hybridized to Probes #1, #2 and #3, again under TMAC conditions. One clone, lambda bP-819, hybridizes to all three probes and is plaque purified and DNA is isolated from a plate lysate. Bacteriophage lambda bP-819 was deposited with the ATCC on June 16, 1987 under accession number 40344. This bP-819 clone encodes the bovine bone growth factor designated bBMP-3.

The region of bP-819 which hybridizes to Probe #2 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IVA. The amino acid sequences corresponding to tryptic Fragments 10 and 12 are underlined. The first underlined sequence corresponds to Fragment 12 while the second corresponds to Fragment 10. This region of bP-819, therefore, which hybridizes to Probe #2 encodes at least 111 amino acids. This amino acid sequence is encoded by the DNA sequence from nucleotide #414 through #746.

EXAMPLE VHuman Bone Inductive FactorsA. hBMP-1

Because the bovine and human bone growth factor genes are presumed to be significantly homologous, the bovine bBMP-1 DNA sequence of Table II (or portions thereof) is used as a probe to screen a human genomic library. The 800bp EcoRI fragment of the bovine genomic clone is labeled with  $^{32}\text{P}$  by nick-translation. A human genomic library (Toole et al., supra) is plated on 20 plates at 40,000 recombinants per plate. Duplicate nitrocellulose filter replicas are made of each plate and hybridized to the nick-translated probe in 5 X SSC, 5 X Denhardt's, 100ug/ml denatured salmon sperm DNA, 0.1% SDS (the standard hybridization solution) at 50 degrees centigrade for approximately 14 hours. The filters are then washed in 1 X SSC, 0.1% SDS at 50 degrees centigrade and subjected to autoradiography. Five duplicate positives are isolated and plaque purified. DNA is obtained from a plate lysate of one of these recombinant bacteriophage, designated LP-H1. LP-H1 was deposited with the ATCC on March 6, 1987 under accession number 40311. This clone encodes at least a portion of the human genomic bone growth factor called hBMP-1. The hybridizing region of LP-H1 is localized to a 2.5kb XbaI/HindIII restriction fragment.

The partial DNA sequence and derived amino acid sequence of lambda LP-H1 are shown below in Table V. The peptide sequence from this clone is 37 amino acids in length and is encoded by the DNA sequence from nucleotide #3440 through nucleotide #3550. The coding sequence of Table V is flanked by approximately 28 nucleotides (a presumptive 5' noncoding sequence) as well as approximately 19 nucleotides (a presumptive 3' noncoding sequence). A comparison of the bBMP-1 sequence of Table II with the hBMP-1 genomic sequence of Table V indicates the significant homology between the two.

Because the size of coding regions and the positions

Human Bone Inductive Factors TABLE V

3419	3429	3439 (1)	3454
CAGCCCCGGC	TTCTTCCTTT	CTCTTTAGCT	GCC TTT CTT GGG GAC ATT GCC CTG GAC
			Ala Phe Leu Gly Asp Ile Ala Leu Asp
			A F L G D I A L D
3469	3484	3499	3514
GAA GAG GAC CTG AGG GCC TTC CAG GTA CAG CAG GCT GTG GAT CTC AGA CGG CAC			
Gl <sub>u</sub> Gl <sub>u</sub> Asp Leu Arg Ala Phe Gln Val Gln Gln Ala Val Asp Leu Arg Arg His			
E E P L R A F Q V Q Q A V D L R K H			
3529	3544 (37)	3560	3570
ACA GCT CGT AAG TCC TCC ATC AAA GCT GCA GGTAAGCGG GTGCCAATGG			
Thr Ala Arg Lys Ser Ser Ile Ile Met			

each plate and hybridized to the nick-translated were 100 fmol cDNA, 5 μl Denhardt's, 100μl ml denatured salmon sperm DNA, 0.1% SDS (the standard hybridization solution), at 56 degrees centigrade for approximately 24 hours. The filter was then washed in 1 X SSC, 0.1% SDS at 56 degrees centigrade and subjected to autoradiography. Five duplicate filters were isolated and plaque purified. DNA was extracted from the lysate of one of these recombinant bacteriophages, designated LP-H1. LP-H1 was deposited with the ATCC on March 1997 under accession number 40311. This clone encodes at least a portion of the human genomic gene growth factor beta 1. The hybridization results of LP-H1 to the human genomic DNA restriction fragment library

The entire nucleotide sequence and derived amino acid sequence of the insert of lambda U2OS-1 is given in Table VI. This cDNA clone encodes a Met followed by a hydrophobic leader sequence characteristic of a secreted protein, and contains a stop codon at nucleotide positions 2226 - 2228. This clone contains an open reading frame of 2190bp, encoding a protein of 730 amino acids with a molecular weight of 83kd based on this amino acid sequence. The clone contains sequence identical to the coding region given in Table V. This protein is contemplated to represent a primary translation product which is cleaved upon secretion to produce the hBMP-1 protein. This clone is therefore a cDNA for hBMP-1 corresponding to human gene fragment contained in the genomic hBMP-1 sequence lambda LP-H1. It is noted that amino acids #550 to #590 of BMP-1 are homologous to epidermal growth factor and the "growth factor" domains of Protein C, Factor X and Factor IX.

Pg. 32 ?

Pg. 34?

The 665 nucleotide sequence at 695 derived amino acid  
 AAG TTC GGC ATT GTG GTC CAC GAG CTG GGC CAC GTC GTC GGC TTC TGG CAC GAA  
 Lys Phe Gly Ile Val Val His Glu Leu Gly His Val Val Gly Phe Trp His Glu  
 K M F G I V V A D E L G H V V G F W H E  
 710 725 740 755  
 CAC ACT CGG CCA GAC CGG GAC CGC CAC GTT TCC ATC GTT CGT GAG AAC ATC CAG  
 His Thr Arg Pro Asp Arg Asp Arg His Val Ser Ile Val Arg Glu Asn Ile Gln  
 H T K P D R D I R H V S I V R E N I Q  
 770 785 800 815  
 CCA GGG CAG GAG TAT AAC TTC CTG AAG ATG GAG CCT CAG GAG GTG GAG TCC CTG  
 Pro Gly Gln Glu Tyr Asn Phe Leu Lys MET Glu Pro Gln Glu Val Glu Ser Leu  
 P G Q E Y W F L K M E P Q E V E S L  
 830 845 860  
 GGG GAG ACC TAT GAC TTC GAC AGC ATC ATG CAT TAC GCT CGG AAC ACA TTC TCC  
 Gly Glu Thr Tyr Asp Phe Asp Ser Ile MET His Tyr Ala Arg Asn Thr Phe Ser  
 G E T D Y D F D S E M H Y A S R N T L F S  
 875 890 905 920  
 AGG GGC ATC TTC CTG GAT ACC ATT GTC CCC AAG TAT GAG GTG AAC GGG GTG AAA  
 Arg Gly Ile Phe Leu Asp Thr Ile Val Pro Lys Tyr Glu Val Asn Gly Val Lys  
 R G I F L D T I V P K Y E V N G V K  
 935 950 965  
 CCT CCC ATT GGC GAA AGG ACA CGG CTC AGC AAG GGG GAC ATT GGC GAA GGC CGC  
 Pro Pro Ile Gly Gln Arg Thr Arg Leu Ser Lys Gly Asp Ile Ala Gln Ala Arg  
 P P G I F Q R T R E L S K G D I A Q A R  
 980 995 1010 1025  
 AAG CTT TAC AAG TGC CCA GCC TGT GGA GAG ACC CTG CAA GAC AGC ACA GGC AAC  
 Lys Leu Tyr Lys Cys Pro Ala Cys Gly Glu Thr Leu Gln Asp Ser Thr Gly Asn  
 K L Y K C P A C G E T L Q P S T G N  
 1040 1055 1070 1085  
 TTC TCC TCC CCT GAA TAC CCC AAT GGC TAC TCT GCT CAC ATG CAC TGC GTG TGG  
 Phe Ser Ser Pro Glu Tyr Pro Asn Gly Tyr Ser Ala His MET His Cys Val Trp  
 F S S P E Y P N G Y S A H M I C V W  
 1100 1115 1130  
 CGC ATC TCT GTC ACA CCC GGG GAG AAG ATC ATC CTG AAC TTC ACG TCC CTG GAC  
 Arg Ile Ser Val Thr Pro Gly Glu Lys Ile Ile Leu Asn Phe Thr Ser Leu Asp  
 R I S V T P G E K T I L N F T S L D  
 1145 1160 1175 1190  
 CTG TAC CGC AGC CGC CTG TGC TGG TAC GAC TAT GTG GAG GTC CGA GAT GGC TTC  
 Leu Tyr Arg Ser Arg Leu Cys Trp Tyr Asp Tyr Val Glu Val Arg Asp Gly Phe  
 L Y R S R L C W Y D Y V E V R D G F  
 1205 1220 1235  
 TGG AGG AAG GCG CCC CTC CGA GGC CGC TTC TGC GGG TCC AAA CTC CCT GAG CCT  
 Trp Arg Lys Ala Pro Leu Arg Gly Arg Phe Cys Gly Ser Lys Leu Pro Glu Pro  
 W R K A F L R G R F C G S K L P E P  
 1250 1265 1280 1295  
 ATC GTC TCC ACT GAC AGC CGC CTC TGG GTT GAA TTC CGC AGC AGC AGC AAT TGG  
 Ile Val Ser Thr Asp Ser Arg Leu Trp Val Glu Phe Arg Ser Ser Ser Asn Trp  
 I V S T D S R L W V E F R S S S N W  
 1310 1325 1340 1355  
 GTT GGA AAG GGC TTC TTT GCA GTC TAC GAA GGC ATC TGC GGG CGT GAT GTG AAA  
 Val Gly Lys Gly Phe Phe Ala Val Tyr Glu Ala Ile Cys Gly Gly Asp Val Lys  
 W G K G F F A V Y E A I C G G D V K

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with the sequence given in Table III at the 3' end of their coding regions, but less so in the more 5' regions. Thus they encode a human protein of similar, though not identical, structure to that above.

Full length hBMP-2 Class I cDNA clones are obtained in the following manner. The 1.5 kb insert of one of the Class II subclones (II-10-1) is isolated and radioactively labeled by nick-translation. One set of the nitrocellulose replicas of the U-2 OS cDNA library screened above (50 filters, corresponding to 1,000,000 recombinant bacteriophage) are rehybridized with this probe under stringent conditions (hybridization at 65° in standard hybridization buffer; washing at 65° in 0.2 X SSC, 0.1% SDS). All recombinants which hybridize to the bovine genomic probe which do not hybridize to the Class II probe are picked and plaque-purified (10 recombinants). Plate stocks are made and small scale bacteriophage DNA preparations made. After subcloning into M13 sequence analysis indicates that 4 of these represent clones which overlap the original Class I clone. One of these, lambda U2OS-39, contains an approximately 1.5 kb insert and was deposited with the ATCC on June 16, 1987 under accession number 40345. The partial DNA sequence (compiled from lambda U2OS-39 and several other hBMP-2 Class I cDNA recombinants) and derived amino acid sequence are shown below in Table VII. Lambda U2OS-39 is expected to contain all of the nucleotide sequence necessary to encode the entire human counterpart of the protein BMP-2 Class II encoded by the bovine gene segment whose partial sequence is presented in Table III. This human cDNA hBMP-2 Class II contains an open reading frame of 1188 bp, encoding a protein of 396 amino acids. This protein of 396 amino acids has a molecular weight of 45kd based on this amino acid sequence. It is contemplated that this sequence represents the primary translation product. The protein is preceded by a 5' untranslated region of 342 bp with stop codons in all frames.

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which the sequence given in Table VII is used, and of the coding regions, but less so in the non-coding regions. They encode a human fibroblast protein.

TABLE VII

10	20	30	40	50	60	70
GTOGACTCTA	GAGTGTGIGT	CAGCACTTGG	CTGGGGACTT	CTTGAACTTG	CAGGGAGAAAT	AACTTGCGCA
80	90	100	110	120	130	140
CCGCCACTTGT	CGCGGGTGOC	TTTGCOCAG	CGGAGCTGTC	TTGGCCATCT	CGAGGCCCCA	CGGCGCGCC
150	160	170	180	190	200	210
ACTCTCTGGC	CTTGCOCAGAC	ACTGAGAOGC	TGTTOOCAGC	GTGAAAAGAG	AGACTGCGCG	CGGGCACCCC
220	230	240	250	260	270	280
GGGAGAAGGA	GGAGGCAAAG	AAAAGGAACG	GAATTCGGT	CCCTGCGCCA	GGTCCTTGA	CCAGAGTTTT
(10 recombinants)	290	300	320	330	340	350
CTTCCATGTTGA	CCCTCTTTCA	ATGGACCGTGT	CCCGCGTGC	TTCCTAGACG	GACTGCGGTC	TCTAAAGGT
415	430	445	460	475	490	505
CGACC ATG GTG GCG ACC CGC TGT CTT CTA GCG TTG CTG CTT CCC CAG GTC	these, MET Val Ala Gly Thr Arg Cys Leu Leu Ala Leu Leu Pro Gln Val	Insert Met Asp Gp	R C L L A L L L P Q V	400	410	420
Leu Leu Gly Gly Ala Ala Gly Leu Val Pro Glu Leu Gly Arg Arg Lys Phe Ala	LL G G A A G E L V P E L G R R K F A	430	445	460	475	490
520	535	550	565	580	595	610
TTC GAG TTG CGG CTG CTC AGC ATG TTC GGC CTG AAA CAG AGA CCC ACC CCC AGC	Ala Ala Ser Ser Gly Arg Pro Ser Ser Gln Pro Ser Asp Glu Val Leu Ser Glu	F E L R L C S M F 6 L K Q R P T P S	505	520	535	550
Arg Asp Ala Val Val Pro Pro Tyr MET Leu Asp Leu Tyr Arg Arg His Ser Gly	R D A V V P P Y M L D L Y R R H S G	565	580	595	610	625
625	640	655	670	685	700	715
CAG CGG CGC TCA CCC GCC CCA GAC CAC CGG TTG GAG AGG GCA GCC AGC CGA CGC	Gln Pro Gly Ser Pro Ala Pro Asp His Arg Leu Glu Arg Ala Ala Ser Arg Ala	AAC ACT GTG CGC AGC TTC CAC CAT GAA GAA TCT TTG GAA GAA CTA CCA GAA ACG	Q P G S P A P D H R L E K A A S R A	605	620	635
Asn Thr Val Arg Ser Phe His His Glu Glu Ser Leu Glu Glu Leu Pro Glu Thr	N T V R S F H E E S L E E L P E T	635	650	665	680	700

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1435            1450            1465            1480  
 GCA TGC TGT GTC CGC ACA GAA CTC AGT GCT ATC TCG ATG CTG TAC CTT GAC GAG  
 Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser MET Leu Tyr Leu Asp Glu  
 A C C U P T E L S A I S M L Y L D E  
 1495            1510            1525  
 AAT GAA AAG GTT GTA TTA AAG AAC TAT CAG GAC ATG GTT GTG GAG GGT TGT GGG  
 Asn Glu Lys Val Val Leu Lys Asn Tyr Gln Asp MET Val Val Glu Gly Cys Gly  
 N E K V V L K N Y Q D M V V E G C G  
 1540 (396)    1553    1563    1573    1583    1593    1603  
 TGT CGC TAGTACAGCA AAATTAAATA CATAAATATA TATATATATA TATATTTAG AAAAAAGAAA  
 Cys Arg  
 C R

AAAA:

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primary translation product.

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747 codon sequence 762 777

AGC CGG GGC AAC ACC GTG AGG AGC TTC CAC CAC GAA GAA CAT CTG GAG AAC ATC  
 Ser Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn Ile  
 S R A N T V R S F H I T E E I L E N I  
 792 807 822 837  
 CCA GGG ACC AGT GAA AAC TCT GCT TTT CGT TTC CTC TTT AAC CTC AGC AGC ATC  
 Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu Ser Ser Ile  
 P G T S E N S A F R P L F N L S S I  
 852 867 882 897  
 CCT GAG AAC GAG GTG ATC TCC TCT GCA GAG CTT CGG CTC TTC OGG GAG CAG GTG  
 Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg Leu Phe Arg Glu Gln Val  
 P E N E V I S S A E L R L F R E E V  
 912 927 942  
 GAC CAG GGC CCT GAT TGG GAA AGG GGC TTC CAC CGT ATA AAC ATT TAT GAG GTT  
 Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile Tyr Glu Val  
 D Q G P D W E R G F H R I N t Y E  
 957 972 987 1002  
 ATG AAG CCC CCA GCA GAA GTG GTG CCT CGG CAC CTC ATC ACA CGA CTA CTG GAC  
 MET Lys Pro Pro Ala Glu Val Val Pro Gly His Leu Ile Thr Arg Leu Leu Asp  
 M K P P A E V V P G H L I T R L L D  
 1017 1032 1047  
 ACG AGA CTG GTC CAC CAC AAT GTG ACA CGG TGG GAA ACT TTT GAT GTG AGC CCT  
 Thr Arg Leu Val His His Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro  
 T R L V H H N V T R W E T F D V S P  
 1062 1077 1092 1107  
 CGG GTC CTT CGC TGG ACC CGG GAG AAG CAG CCA AAC TAT GGG CTA GCC ATT GAG  
 Ala Val Leu Arg Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu  
 A V L R W T R E K Q P N Y G L A I E  
 1122 1137 1152 1167  
 GTG ACT CAC CTC CAT CAG ACT CGG ACC CAC CAG GGC CAG CAT GTC AGG ATT AGC  
 Val Thr His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser  
 V T H L H Q T R T H Q G Q H V R I S  
 1182 1197 1212  
 CGA TCG TTA CCT CAA GGG AGT GGG AAT TGG GCC CAG CTC CGG CCC CTC CTG GTC  
 Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu Val  
 R S L P Q G S G N W A Q L R P L L V  
 1227 1242 1257 1272  
 ACC TTT GGC CAT GAT GGC CGG GGC CAT GCC TTG ACC CGA CGC CGG AGG GGC AAG  
 Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg Arg Ala Lys  
 T F G H D G R G H A L T R R R R A K  
 1287 1302 1317  
 CGT AGC CCT AAG CAT CAC TCA CAG CGG GCC AGG AAG AAG AAT AAG AAC TGC CGG  
 Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys Arg  
 R S P K H H S Q R A R K K N K N C R  
 1332 1347 1362 1377  
 CGC CAC TCG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC TGG ATT GTG  
 Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val  
 R H S L Y V D F S D V G W N D W I V  
 1392 1407 1422 1437  
 GCC CCA CCA GGC TAC CAG GGC TTC TAC TCC CAT GGG GAC TGC CCC TTT CCA CTG  
 Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp Cys Pro Phe Pro Leu  
 A P P G Y Q A F Y C H G D C P F P L

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The sequences of BMP-2 Class I and II, as well as BMP-3 as shown in Tables III, IV, VII and VIII have significant homology to the beta (B) and beta (A) subunits of the inhibins. The inhibins are a family of hormones which are presently being investigated for use in contraception. See, A. J. Mason et al, Nature, 318:659-663 (1985). To a lesser extent they are also homologous to Mullerian inhibiting substance (MIS), a testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo and transforming growth factor-beta (TGF- $\beta$ ) which can inhibit or stimulate growth of cells or cause them to differentiate. Furthermore, the sequence of Table VII encoding hBMP-2 Class II has significant homology to the Drosophila decapentaplegic (DPP-C) locus transcript. See, J. Massague, Cell, 49:437-438 (1987); R. W. Padgett et al, Nature, 325:81-84 (1987); R. L. Cathey et al, Cell 45:685-698 (1986). It is considered possible therefore that BMP-2 Class II is the human homolog of the protein made from this transcript from this developmental mutant locus.

#### C. BMP-3

Because bovine and human bone growth factor genes are presumed to be significantly homologous, oligonucleotide probes which have been shown to hybridize to the bovine DNA sequence of Table IV.A and IV.B are used to screen a human genomic library. A human genomic library (Toole et al., supra) is screened using these probes, and presumptive positives are isolated and DNA sequence obtained as described above. Evidence that this recombinant encodes a portion of the human bone inductive factor molecule relies on the bovine/human protein and gene structure homologies.

Once a recombinant bacteriophage containing DNA encoding a portion of the human BMP-3 molecule is obtained the human coding sequence is used as a probe as described in Example V (A) to identify a human cell line or tissue which synthesizes BMP-3. mRNA is selected by oligo (dT) cellulose

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VIII or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3, pJL4 [Geough et al., EMBO J., 4:645-653 (1985)]. The transformation of these vectors into appropriate host cells can result in expression of osteoinductive factors. One skilled in the art could manipulate the sequences of Tables II-VIII by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). The modified bone inductive factor coding sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and bone inductive factor expressed thereby. For a strategy for producing extracellular expression of bone inductive factor in bacterial cells, see, e.g., European patent application EPA 177,343. See also, e.g., the procedures. Similar manipulations can be performed for the construction of an insect vector [See, e.g. procedures described in published European patent application 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO86/00639 and European patent application EPA 123,289].

A method for producing high levels of an osteoinductive factor of the invention from mammalian cells involves the construction of cells containing multiple copies of the heterologous bone inductive factor gene. The heterologous gene

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Danna et al. PNAS 78:7575-7578 (1981); Luthman and Magnusson, Nucl. Acids Res. 11: 1295-1308 (1983)]. Serum-free 24 hr. conditioned medium is collected from the cells starting 40-70 hr. post-transfection.

The mammalian expression vector pMT2 Cla-Xho (pMT2 CX) is a derivative of p91023 (b) (Wong et al., Science 228:810-815, 1985) differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline-resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 Cla-Xho have been described (Kaufman, R.J., 1985; Proc. Natl. Acad. Sci. USA 82:689-693) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in *E. coli*.  
and some inducible fusions will be used.

Plasmid pMT2 Cla-Xho is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform *E. coli* HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2CX is then constructed by digesting pMT2 with Eco RV and XbaI, treating the digested DNA with Klenow fragment of DNA polymerase I, and ligating Cla linkers (NEBiolabs, CATCGATG). This removes bases 2266 to 2421 starting from the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. Plasmid DNA is then digested with EcoRI, blunted as above, and ligated to an EcoRI adapter,

5' PO<sub>4</sub>-AATTCCCTCGAGAGCT 3'

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Ianna. Addition of human BMP-1 to the matrix material resulted in formation of cartilage-like nodules at 7 days post implantation. The chondroblast-type cells were recognizable by shape and expression of metachromatic matrix. The amount of activity observed for human BMP-1 was dependent upon the amount of human BMP-1 protein added to the matrix. Table IX illustrates the dose-response relationship of human BMP-1 protein to the amount of bone induction observed.

See also Example 1, Table IX for the Table IX.

<u>IMPLANT NUMBER</u>	<u>AMOUNT USED</u> (equivalent of ml HepG2 cells in transfection media)	<u>HISTOLOGICAL SCORE</u>
876-134-1	10 <sup>-1</sup> BMP-1	C+2
876-134-2	3 BMP-1	C+1
876-134-3	1 BMP-1	C +/-
876-134-4	10 MOCK	C -
876-134-5	3 MOCK	C -
876-134-6	plasmid alone MOCK	no induction seen C -

Cartilage (c) activity was scored on a scale from 0(-) to 5. Culture Collection ATCC, Rockville, MD.

Similar levels of activity are seen in the Heparin Sepharose fractionated COS cell extracts. Partial purification is accomplished in a similar manner as described above except that 6 M urea is included in all the buffers. Further, in a rat bone formation assay as described above, BMP-2 has similarly demonstrated chondrogenic activity.

The procedures described above may be employed to isolate other bone inductive factors of interest by utilizing the bovine bone inductive factors and/or human bone inductive factors as a probe source. Such other bone inductive factors may find similar utility in, inter alia, fracture repair.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications

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International Application No: PCT/

## MICROORGANISMS

**Optional Sheet in connection with the microorganism referred to on page \_\_\_\_\_, line \_\_\_\_\_ of the description<sup>8</sup>**

**A. IDENTIFICATION OF DEPOSITS**

Further deposits are identified on an additional sheet

amount of human BMP-4 protein added to the matrix. American Type Culture Collection

**Address of depository institution (including postal code and country) \***

12301 Parklawn Drive  
Rockville, Maryland 20852 USA

<u>Name of Deposit</u>	<u>ATCC No.</u>	<u>Referred to on page/line</u>	<u>Date of Deposit</u>
LP-H1	40311	29/20	March 4, 1987
bP50	40295	20/3	December 15, 1986
bP-21	40310	22/18	March 4, 1987
U2OS-3	40342	44/22	June 16, 1987
Lambda U2-OS-1	40343	32/33	June 16, 1987
Lambda BP819	40344	25/23	June 16, 1987
U2OS-39	40345	39/21	June 16, 1987

**6. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE. (This section applies to all companies. Similar sections apply to other sections of the form.)**

Certificate of accuracy and correctness of the above copy of the original document.

D. SEPARATE FURNISHING OF INDICATIONS : (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later\* (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E.  This sheet was received with the international application when filed (to be checked by the receiving Office)

*Theodis Singlet*  
(Authorized Officer)

(Authorized Officer)

The date of receipt (from the applicant) by the International Bureau 14

(Authorizing Officer)

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with a DNA sequence encoding BMP-2 Class I, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class I from said culture medium.

12. A process for according to Claim 11 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VII.

13. A process for producing BMP-2 Class II comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class II, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class II from said culture medium.

14. A process according to Claim 13 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VIII.

15. A process for producing BMP-3 comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-3, said DNA sequence being in relative association with an expression control sequence therefor and isolating BMP-3 from said culture medium.

16. A process according to Claim 15 wherein said DNA sequence comprises substantially the nucleotide sequence of Table IVA and IVB.

17. A cDNA sequence encoding BMP-1 comprising substantially the nucleotide sequence of Table VI or a sequence which hybridize thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-1.

18. A cDNA sequence encoding BMP-2 Class I comprising substantially the nucleotide sequence of Table VII or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class I.

19. A cDNA sequence encoding BMP-2 Class II comprising

**AMENDED CLAIMS**

[received by the International Bureau on 8 December 1987 (08.12.87)  
original claims 6, 8, 10, 12, 14, 16-20 amended;  
new claims 21-23 added; other claims unchanged (13 pages)]

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1. A pharmaceutical composition comprising a protein selected from the group consisting of:  
(a) BMP-1;  
(b) BMP-2 Class I;  
(c) BMP-2 Class II;  
(d) BMP-3; and  
mixtures thereof, in a pharmaceutically acceptable vehicle.
2. A composition of Claim 1 wherein said protein is BMP-1.
3. A composition of Claim 1 wherein said protein is BMP-2 Class I.
4. A composition of Claim 1 wherein said protein is BMP-2 Class II.
5. A composition of Claim 1 wherein said protein is BMP-3.
6. The pharmaceutical composition of Claim 1 further comprising a matrix capable of delivering the composition to the site of the bone or cartilage defect and providing a structure for inducing bone or cartilage formation.
7. The composition of Claim 6 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.
8. A method for inducing bone or cartilage formation in a patient in need of same comprising administering to said patient an effective amount of a composition of Claim 1-7.

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Received by the International Bureau  
on 11.01.1988  
in accordance with Article 17.17 of the  
Rules of Procedure of the International  
Bureau.

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pna.m560eul... 565 575 585 590

GAC GAG GAC AGC TAT ATT GTG TTC ACC TAT CGA CCT TGC GGG TGC TGC TGC TAC  
Asp Glu Asp Ser Tyr Ile Val Phe Thr Tyr Arg Pro Cys Gly Cys Cys Ser Tyr  
DMP

605	620	635	650
GTG GGT CGC CGC GGG GGC CCC CAG GCC ATC TCC ATC GGC AAG AAC TGT GAC			
Val Gly Arg Arg Gly Gly Pro Gln Ala Ile Ser Ile Gly Lys Asn Cys Asp			

665	680	695	
AAG TTC GGC ATT GTG GTC CAC GAG CTG GGC CAC GTC GTC GGC TTC TGG CAC GAA			
Lys Phe Gly Ile Val Val His Glu Leu Gly His Val Val Gly Phe Trp His Glu			

710	725	740	755
CAC ACT CGG CCA GAC CGG GAC CGC CAC GTT TCC ATC GTT CGT GAG AAC ATC CAG			
His Thr Arg Pro Asp Arg Asp His Val Ser Ile Val Arg Glu Asn Ile Gln			

770	785	800	815
CCA GGG CAG GAG TAT AAC TTC CTG AAG ATG GAG CCT CAG GAG GTG GAG TCC CTG			
Pro Gly Gln Glu Tyr Asn Phe Leu Lys MET Glu Pro Gln Glu Val Glu Ser Leu			

830	845	860	
GGG GAG ACC TAT GAC TTC GAC AGC ATC ATG CAT TAC GCT CGG AAC ACA TTC TCC			
Gly Glu Thr Tyr Asp Phe Asp Ser Ile MET His Tyr Ala Arg Asn Thr Phe Ser			

875	890	905	920
AGG GGC ATC TTC CTG GAT ACC ATT GTC CCC AAG TAT GAG GTG AAC GGG GTG AAA			
Arg Gly Ile Phe Leu Asp Thr Ile Val Pro Lys Tyr Glu Val Asn Gly Val Lys			

935	950	965	
CCT CCC ATT GGC CAA AGG ACA CGG CTC AGC AAG GGG GAC ATT GCC CAA GCC CGC			
Pro Pro Ile Gly Gln Arg Thr Arg Leu Ser Lys Gly Asp Ile Ala Gln Ala Arg			

980	995	1010	1025
AAG CTT TAC AAG TGC CCA GCC TGT GGA GAG ACC CTG CAA GAC AGC ACA GGC AAC			
Lys Leu Tyr Lys Cys Pro Ala Cys Gly Glu Thr Leu Gln Asp Ser Thr Gly Asn			

1040	1055	1070	1085
TTC TCC TCC CCT GAA TAC CCC AAT GGC TAC TCT GCT CAC ATG CAC TGC GTG TGG			
Phe Ser Ser Pro Glu Tyr Pro Asn Gly Tyr Ser Ala His MET His Cys Val Trp			

1100	1115	1130	
CGC ATC TCT GTC ACA CCC GGG GAG AAG ATC ATC CTG AAC TTC ACG TCC CTG GAC			
Arg Ile Ser Val Thr Pro Gly Glu Lys Ile Ile Leu Asn Phe Thr Ser Leu Asp			

1145	1160	1175	1190
CTG TAC CGC AGC CGC CTG TGC TGG TAC GAC TAT GTG GAG GTC CGA GAT GCC TTC			
Leu Tyr Arg Ser Arg Leu Cys Trp Tyr Asp Tyr Val Glu Val Arg Asp Gly Phe			

1205	1220	1235	
TGG AGG AAG GCG CCC CTC CGA GCC CGC TTC TGC GGG TCC AAA CTC CCT GAG CCT			
Trp Arg Lys Ala Pro Leu Arg Gly Arg Phe Cys Gly Ser Lys Leu Pro Glu Pro			

1955	1970	1985	2000			
GAG GGC AAT GAT GTG TCC AAG TAC GAC TTC GTG GAG GTG CGC AGT GGA CTC ACA						
Glu Gly Asn Asp Val Cys Lys Tyr Asp Phe Val Glu Val Arg Ser Gly Leu Thr						
Asp Glu Asp Ser Tyr Ile Val Phe Thr Tyr Asn Ile Val Glu Ser Asn Ser Ser Ser						
2015	2030	2045				
GCT GAG TOC AAG CTG CAT GGC AAG TTC TGT CGT TCT GAG AAG CCC GAG GTC ATC						
Ala Asp Ser Lys Leu His Gly Lys Phe Cys Gly Ser Glu Lys Pro Glu Val Ile						
2060	2075	2090	2105			
ACC TOC CAG TAC AAC AAC ATG CGC GTG GAG TTC AAG TCC GAC AAC ACC GTG TCC						
Thr Ser Gln Tyr Asn Asn MET Arg Val Glu Phe Lys Ser Asp Asn Thr Val Ser						
2120	2135	2150	2165			
AAA AAG GGC TTC AAG GCC CAC TTC TCA GAA AAG AGG CCA GCT CTG CAG CCC						
Lys Lys Gly Phe Lys Ala His Phe Phe Ser Glu Lys Arg Pro Ala Leu Gln Pro						
2180	2195	2210				
CCT CGG GGA CGC CCC CAC CAG CTC AAA TTC CGA GTG CAG AAA AGA AAC CGG ACC						
Pro Arg Gly Arg Pro His Gln Leu Lys Phe Arg Val Gln Lys Arg Asn Arg Thr						
2225	2235	2245	2255	2265	2275	2285
CCC CAG TGAGGCCCTGC CAGGCCCTCCC GGACCCCTTG TTACCTAGGA ACCTCACCTT GGACCGAACG						
Pro Gln						
2295	2305	2315	2325	2335	2345	2355
GGATGGGGGC TTGGGTGGCC ACCAACCCCC CACCTCCACT CTGCCATTCC GGCCACCCCTC CCTCTGGCG						
Arg						
2365	2375	2385	2395	2405	2415	2425
GACAGAACIG GIGCTCTCTT CTCCCCACTG TGCCCGTCCG OGGACCGGGG ACCCTCCCCC GIGCCCTACC						
Arg						
2435	2445	2455	2465	2475	2485	2495
CCCTCCCAIT TTGATGGTGT CTGTGACAIT TOCTGTGIG AAGTAAAAGA GGGACCCCTG CGTCTGGCT						
Arg CTT TAC AAG TGC GCA GGC TGT GCA GAG AGG CTC GCA GAT TAC GGC TAA						
CTAGA						

11. A process for producing BMP-2 Class I comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class I, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class I from said culture medium.

12. A process for according to Claim 11 wherein said DNA sequence comprises substantially the nucleotide sequence as

pg 6b

1150 850 890 865 880  
 TTA GGA AAC AAT AGC AGT TTC CAT CAC CGA ATT AAT ATT TAT GAA ATC ATA AAA  
 Leu Gly Asn Asn Ser Ser Phe His His Arg Ile Asn Ile Tyr Glu Ile Ile Lys  
  
 895 2015 910 925 940  
 CCT GCA ACA GCC AAC TCG AAA TTC CCC GTG AEC AGT CTT TTG GAC ACC AGG TTG  
 Pro Ala Thr Ala Asn Ser Lys Phe Pro Val Thr Ser Leu Leu Asp Thr Arg Leu  
  
 955 970 985  
 GTG AAT CAG AAT GCA AGC AGG TGG GAA AGT TTT GAT GTC ACC CCC GCT GTG ATG  
 Val Asn Gln Asn Ala Ser Arg Trp Glu Ser Phe Asp Val Thr Pro Ala Val MET  
  
 1000 1015 1030 1045  
 CGG TGG ACT CCA CAG GGA CAC GCC AAC CAT GGA TTC GTG GTG GAA GTG GCC CAC  
 Arg Trp Thr Ala Gln Gly His Ala Asn His Gly Phe Val Val Glu Val Ala His  
  
 1060 1075 1090 1105  
 TTG GAG GAG AAA CAA GGT GTC TCC AAG AGA CAT GTT AGG ATA AGC AGG TCT TTG  
 Leu Glu Glu Lys Gln Gly Val Ser Lys Arg His Val Arg Ile Ser Arg Ser Leu  
  
 1120 1135 1150  
 CAC CAA GAT GAA CAC AGC TGG TCA CAG ATA AGG CCA TTG CTA GTA ACT TTT GGC  
 His Gln Asp Glu His Ser Trp Ser Gln Ile Arg Pro Leu Leu Val Thr Phe Gly  
  
 1165 1180 1195 1210  
 CAT GAT GGA AAA GGG CAT CCT CTC CAC AAA AGA GAA AAA CGT CAA GCC AAA CAC  
 His Asp Gly Lys His Pro Leu His Lys Arg Glu Lys Arg Gln Ala Lys His  
  
 1225 1240 1255  
 AAA CAG CGG AAA CGC CCT AAG TCC AGC TTG AAG AGA CAC CCT TTG TAC GTG GAC  
 Lys Gln Arg Lys Arg Leu Lys Ser Ser Cys Lys Arg His Pro Leu Tyr Val Asp  
  
 1270 1285 1300 1315  
 TTC AGT GAC GTG GGG TGG AAT GAC TGG ATT GTG GCT CCC CGG TAT CAC GCC  
 Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr His Ala  
  
 1330 1345 1360 1375  
 TTT TAC TGC CAC GGA GAA TGC CCT TTT CCT CTG GCT GAT CAT CTG AAC TCC ACT  
 Phe Tyr Cys His Gly Glu Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr  
  
 1390 1405 1420  
 AAT CAT GCC ATT GTT CAG ACG TTG GTC AAC TCT GTT AAC TCT AAG ATT CCT AAG  
 Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys  
  
 1435 1450 1465 1480  
 GCA TGC TGT GTC CGC ACA GAA CTC AGT GCT ATC TCG ATG CTG TAC CCT GAC GAG  
 Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser MET Leu Tyr Leu Asp Glu

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522 537 552 567  
 GGC GAC GCG CGA CGA CGC CTC TCA GGG CAG AGC CAT GAG CTC CTG CCG GAC TTC  
 Gly His Ala Gly Gly Arg Arg Ser Gly Gln Ser His Glu Leu Leu Arg Asp Phe

582 597 612 627  
 GNG GCG ACA CTT CTG CAG ATG TTT GGG CTG CGC CGC CGC CCG CAG CCT AGC AAG  
 Glu Ala Thr Leu Leu Gln MET Phe Gly Leu Arg Arg Pro Gln Pro Ser Lys

642 657 672  
 AGT GCC GTC ATT CGG GAC TAC ATG CGG GAT CTT TAC CGG CTT CAG TCT GGG GAG  
 Ser Ala Val Ile Pro Asp Tyr MET Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu

687 702 717 732  
 GAG GAG GAA GAG CAG ATC CAC AGC ACT GGT CTT GAG TAT CCT GAG CGC CGC GCC  
 Glu Glu Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala

747 762 777  
 AGC CGG GCC AAC ACC GTG AGG AGC TTC CAC CAC GAA GAA CAT CTG GAG AAC ATC  
 Ser Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Asn Ile

792 807 822 837  
 CCA GGG ACC AGT GAA AAC TCT GCT TTT CGT TTC CTC TTT AAC CTC AGC AGC ATC  
 Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu Ser Ser Ile

852 867 882 897  
 CCT GAG AAC GAG CGG ATC TCC TCT GCA GAG CTT CGG CTC TTC CGG GAG CAG GTG  
 Pro Glu Asn Glu Ala Ile Ser Ser Ala Glu Leu Arg Leu Phe Arg Glu Gln Val

912 927 942  
 GAC CAG GGC CCT GAT TGG GAA AGG CGC TTC GAC CGT ATA AAC ATT TAT GAG GTT  
 Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile Tyr Glu Val

957 972 987 1002  
 ATG AAG CCC CCA GCA GAA GTG GTG CCT GGG CAC CTC ATC ACA CGA CTA CTG GAC  
 MET Lys Pro Pro Ala Glu Val Val Pro Gly His Leu Ile Thr Arg Leu Leu Asp

1017 1032 1047  
 ACG AGA CTG GTC CAC CAC AAT GTG ACA CGG TGG GAA ACT TTT GAT GTG AGC CCT  
 Thr Arg Leu Val His His Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro

1062 1077 1092 1107  
 GCG GTC CTT CGC TGG ACC CGG CGG CAG AAG CCA AAC TAT GGG CTA CGC ATT GAG  
 Ala Val Leu Arg Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu

1122 1137 1152 1167  
 GTG ACT CAC CTC CAT CAG ACT CGG ACC CAC CAG GGC CAG CAT GTC AGG ATT AGC  
 Val Thr His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser

1182 1197 1212  
 CGA TOG TTA CCT CAA GGG AGT GGG AAT TGG GCC CAG CTC CGG CCC CTC CTG GTC  
 Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu Val

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... association with an expression control sequence therefor and isolating BMP-3 from said culture medium.

16. A process according to Claim 15 wherein said DNA sequence comprises substantially the nucleotide sequence as follows:

383 393 403 413 428  
 GAGGAGGAAG CGGTCTAOGG GGGTCCCTCT CCTCTGCA G AAC AAT GAG CTT CCT GGG GCA  
 Asn Asn Glu Leu Pro Gly Ala

443                    458                    473                    488  
GAA TAT CAG TAC AAG GAG GAT GAA GTC TGG GAG GAG AGG AAG CCT TAC AAG ACT  
Glu Tyr Gln Tyr Lys Glu Asp Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr

503 . 518 533  
 CTT CAG ACT CAG CCC CCT GAT AAG AGT AAG AAC AAA AAG AAA CAG AGG AAG GGA  
 Leu Gln Thr Gln Pro Pro Asp Lys Ser Lys Lys Asn Lys Lys Gln Arg Lys Gly

548 563 578 593  
CCT CAG CAG AAG AGT CAG ACG CTC CAG TTT GAT GAA CAG ACC CTG AAG AAG GCA  
Pro Gln Gln Lys Ser Gln Thr Leu Gln Phe Asp Glu Gln Thr Leu Lys Lys Ala

608 623 638  
 AGA AGA AAG CAA TGG ATT GAA CCC CGG AAT TGT GCG AGA OGG TAC CTT AAA GTG  
 Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn Cys Ala Arg Arg Tyr Leu Lys Val

653 668 683 698  
 GAC TTC GCA GAT ATT GGC TGG AGC GAA TGG ATT ATT TCC CCC AAG TCC TTC GAT  
 Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile Ile Ser Pro Lys Ser Phe Asp

713                    728                    743                    756                    766  
 GCC TAT TAC TGC TOC GGA GCG TCC CAG TTC CCC ATG CCA AAG GTAGGCCATTG TTTTTTGTC  
 Ala Tyr Tyr Cys Ser Gly Ala Cys Glu Phe Pro Met Pro Lys Ser  
 ET Lys Pro Pro Ala Glu Val Val Pro Glu His Leu Ile Val

## 776 786

### SILICOCHILOUS ATTHOCANTHUS · and

284 294 304 319  
TAACCTGTTTCCTCCCTT TCGTTCTTAG TCT TTG AAG CCA TCA AAT CAC GCT ACC  
Ser Leu Lys Pro Ser Asn His Ala Thr

34 349 364 379  
TC CAG AGT ATA GTG AGA GCT GTG CGG GTC GTC CCT GGA ATC CCC GAG CCT TGC  
le Gln Ser Ile Val Arg Ala Val Gly Val Val Pro Gly Ile Pro Glu Pro Cys

394 409 424 439  
 GT GTG CCA GAA AAG ATG TCC TCA CTC ACC ATC TTA TTC TTT GAT GAA AAC AAG  
 ys Val Pro Glu Lys MET Ser Ser Leu Ser Ile Leu Phe Phe Asp Glu Asp Lys

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